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Detection and molecular status of *Isospora* sp. from the domestic pigeon (*Columba livia domestica*).

Ryuma Matsubara

Thomas Jefferson University; Tohoku University, Ryuma.Matsubara@jefferson.edu

Yasuhiro Fukuda

Tohoku University

Fumi Murakoshi

Tohoku University

Osamu Nomura

Ushihama Pet Clinics

Toru Suzuki

Odagawa Animal Hospital

See next page for additional authors

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Authors

Ryuma Matsubara, Yasuhiro Fukuda, Fumi Murakoshi, Osamu Nomura, Toru Suzuki, Chika Tada, and Yutaka Nakai

1 **Title:**

2 Detection and molecular status of *Isospora* sp. from the domestic pigeon (*Columba*
3 *livia domestica*)

4

5 **Authors:** Ryuma Matsubara^a, Yasuhiro Fukuda^a, Fumi Murakoshi^a, Osamu Nomura^b,
6 Toru Suzuki^c, Chika Tada^a, Yutaka Nakai^{a*} (*: Corresponding author)

7

8 **Affiliations and addresses of the authors:**

9 a: Laboratory of Sustainable Environmental Biology, Graduate School of

10 Agricultural Science, Tohoku University. 232-3 Yomogida, Naruko-Onsen, Osaki,

11 Miyagi 989-6711, Japan.

12 b: Ushihama Pet Clinics, 2547-11 Ninomiya, Kumagawa, Fussa, Tokyo 197-0002,

13 Japan.

14 c: Odagawa Animal Hospital, 2129 Noborito, Tama, Kawasaki, Kanagawa 214-0014,

15 Japan.

16

17 **E-mail address, telephone and fax numbers of the corresponding author:**

18 E-mail: nakai@bios.tohoku.ac.jp

19 Telephone/Fax: +81-229-84-7391

20

21 **Abstract**

22 The domestic pigeon, *Columba livia domestica*, is reared for meat
23 production, as a pet, or for racing. Few reports have characterized the parasitic
24 protists from the genus *Isospora* isolated from Columbiformes. We detected
25 *Isospora*-like oocysts from *C. livia* reared for racing. The oocyst contained two
26 sporocysts, and each sporocyst included four sporozoites. The sporulated oocysts
27 (n=4) were spherical; their mean diameters were 25.6 (24.0–27.2) × 24.7 (23.4–26.0)
28 μm. Micropyles, polar granules, and oocyst residuum were absent. The mean length
29 and width of the sporocysts (n=8) were 19.5 (18.5–20.5) and 11.2 (10.2–12.1) μm,
30 respectively. Stieda and sub-Stieda bodies were observed. Single-oocyst PCR
31 revealed two different 18S rRNA gene sequences and one 28S rRNA gene sequence
32 in a single oocyst of *Isospora* sp. Based on a phylogenetic analysis of the 18S rRNA
33 gene, the two sequences made a group which fell within a cluster of known avian
34 *Isospora* species. A tree based on the 28S rRNA gene sequence indicated that
35 sequences from the pigeon *Isospora* sp. fell within a cluster of avian *Isospora*
36 species. Both trees failed to clarify the phylogenetic relationships among the avian

37 *Isospora* species due to limited resolution. Because the morphological description of
38 *Isospora* sp. is based on only four oocysts, *Isospora* sp. is not proposed as a novel
39 species here. This is the first description of *Isospora* sp. isolated from the domestic
40 pigeon *C. livia*.

41

42 **Keywords:**

43 *Isospora* sp., *Columba livia domestica*, domestic pigeon, coccidia, *Atoxoplasma*
44 *columbae*

45

46 The genus *Isospora* (Coccidia: Eimeriidae) comprises parasitic protists
47 belonging to the phylum Apicomplexa and is distributed worldwide. Coccidian
48 parasites are highly host specific and taxonomic studies have relied mostly on host
49 specificity [1]. The domestic pigeon, *Columba livia domestica*, is reared for meat
50 production, as pets, and for racing. Coccidian parasites are important pathogens in
51 pigeon rearing because the parasites cause intestinal disease, such as diarrhea, and
52 are sometimes lethal [2]. Two *Eimeria* species have been characterized as causative
53 agents of coccidiosis in pigeons, while no *Isospora* species have been isolated from
54 *C. livia* [3]. Recently, we found *Isospora*-like oocysts detected in the feces of *C.*
55 *livia*. In this report, we describe morphological characteristics and perform a
56 phylogenetic analysis of the *Isospora* sp. from *C. livia* based on the single-oocyst
57 method.

58 Unsporulated coccidian oocysts were obtained from droppings gathered from
59 16 domestic pigeons reared in Tokyo, Japan, and kept at 4°C within a humid tube.
60 To identify these oocysts, we performed sporulation by referring to a standard
61 technique for avian coccidia [4] equipping some modifications. In brief, the feces

62 were placed in 2.5% (w/v) potassium dichromate solution ($K_2Cr_2O_7$) and incubated
63 at 25°C for 5 days under aerobic conditions. The material was then passed through a
64 fine mesh and the oocysts were purified by sucrose gradient centrifugation [5] and
65 identified. In a result, *Eimeria* oocysts were dominant and oocysts of *Isospora* sp.
66 were hardly detectable. We identified the *Eimeria* oocysts as *E. labbeana* [6] and *E.*
67 *columbarum* [7] based on morphological features. Despite *Isospora* sp. was less
68 frequent in the population, we could identify these based on the size, and the number
69 of sporocysts per oocyst. The purified oocysts were stored at 4°C in
70 phosphate-buffered saline until examination.

71 Sporulated oocysts were suspended in Milli-Q purified water (Millipore,
72 Billerica, MA, USA). Diluted drops containing oocysts were placed on plastic
73 dishes. Each *Isospora* oocyst was isolated from the drops under an inverted
74 microscope (TMD300; Nikon, Tokyo, Japan) using a micropipette or glass capillary.
75 To eliminate additional oocysts as contamination picked up oocysts were passed
76 through several water drops, and we checked contamination at each step by
77 microscopic observation. The isolated oocysts were individually placed onto a cover

78 glass and verified by thorough observation, and were then photographed using a
79 differential interference contrast inverted microscope (TMD300) with a $\times 40$
80 objective lens. In total, four oocysts were observed for the morphological
81 description.

82 A representative oocyst is shown in Fig. 1. Table 1 summarizes the
83 morphological features of four isolated oocysts and eight isolated sporocysts. The
84 oocysts were spherical, consisted of two sporocysts, and each sporocyst included
85 four sporozoites. The mean oocyst diameters were $25.6 (24.0\text{--}27.2) \times 24.7 (23.4\text{--}$
86 $26.0) \mu\text{m}$, and the length/width (L/W) ratio was 0.99–1.1. The monolayer wall was
87 $\sim 1.5 \mu\text{m}$ thick. The oocysts were light pink and with a rough surface. The average
88 diameters of the sporocysts were $19.5 (18.5\text{--}20.5) \times 11.2 (10.2\text{--}12.1) \mu\text{m}$,
89 respectively, the L/W ratio was 1.6–2.0, and the wall was $\sim 0.5 \mu\text{m}$ thick. The texture
90 was that of a single, smooth layer. The Stieda body was nipple-like and $1.8 (1.4\text{--}2.2)$
91 μm in width, the sub-Stieda body was $5.1 (4.6\text{--}5.6) \times 2.9 (2.6\text{--}3.2) \mu\text{m}$ in width, and
92 the sporocyst residuum consisted of loosely clustered homogeneous granules of
93 various sizes. The sporozoites (n=16) contained two refractile bodies: the smaller at

94 the apical end and the larger at the posterior end. None of the pigeons presented with
95 clinical symptoms in the poultry house; therefore, the *Isospora* sp. would be
96 nonpathogenic or rarely pathogenic. However, to address the pathogenicity, an
97 experimental infection to pathogen-free pigeons will be required.

98 We then reviewed the literature on *Isospora* spp. infecting birds of the genus
99 *Columba* or related genera. *Isospora gallicolumbae* was previously isolated from
100 Beccari's ground dove, *Gallicolumba beccarii*, in Papua New Guinea [8]. We
101 compared the taxonomic information of *I. gallicolumbae* with that of the *Isospora* sp.
102 from domestic pigeons. The reported sizes of oocysts and sporocysts of *I.*
103 *gallicolumbae* are $16.0 \times 20.0 \mu\text{m}$ and $8.0 \times 12.0 \mu\text{m}$, respectively. These sizes are
104 smaller than those of the *Isospora* sp. detected in this study. Furthermore, sporocysts
105 of *I. gallicolumbae* possess no sub-Stieda bodies, while such structures were clearly
106 observed in the pigeon *Isospora* sp. (Fig. 1). *Gallicolumba beccarii* and *C. livia*
107 belong to different genera in the family Columbidae, suggesting different host ranges
108 of *I. gallicolumbae* and the pigeon *Isospora* sp. due to the strict host specificity of
109 isosporoid species. In addition, we observed that the morphological features of the

110 pigeon *Isospora* sp. differed in many ways from *I. gallicolumbae*. Thus, the pigeon
111 *Isospora* sp. is morphologically and biologically distinguishable from *I.*
112 *gallicolumbae*.

113 After recording the morphological features, each oocyst was subjected to
114 single-oocyst polymerase chain reaction (PCR) according to previously described
115 methods with modifications [9]. Prior to freeze-fracture we carefully observed drops
116 again and verified no additional oocyst existed. Each isolated oocyst was transferred
117 to a PCR tube; the tubes were then subjected to five freeze-thaw cycles to rupture the
118 oocyst wall. To improve the efficiency of PCR amplification, we used a total
119 genomic DNA amplification technique with a GenomiPhi V2 Whole-Genome
120 Amplification Kit (GE Healthcare, Tokyo, Japan). The enriched DNA product was
121 diluted 10–100-fold with Milli-Q water and used as the PCR template. The 18S
122 rRNA gene was amplified with the primers E18SF and E18SR [10], and the 28S
123 rRNA gene was amplified with the forward and reverse primers E28S-4Fw
124 (5'-GACCTGAAATCAGTCGAGGTTAC-3') and E28S-974Rv
125 (5'-CTTGGTCCGTGTTTCAAGACGC-3'), respectively. The primers targeting the

126 28S rRNA gene (corresponding to positions 4–974 bp of *Eimeria tenella*) were
127 designed manually based on an alignment of the 5' region of the 28S rRNA gene
128 sequences of related species *Eimeria tenella* (AF026388), *E. alabamensis*
129 (AF076861), *Atoxoplasma* sp. (AY283869), *Toxoplasma gondii* (AF076901,
130 L25635), *Plasmodium falciparum* (U48228), and *Cryptosporidium parvum*
131 (AF040725).

132 We subjected all four oocysts to single-oocyst PCR; however, for both genes
133 PCR amplification was successful for only one same oocyst. The low efficiency of
134 PCR amplification was probably due to the small amount of genomic DNA in a
135 single oocyst. Because direct sequencing was not able to clearly determine the
136 sequence of the 18S rRNA gene, which exhibited heterogeneity, we used the
137 standard TA cloning technique prior to sequencing and obtained two sequences. In
138 contrast, the nucleotide sequence of the 28S rRNA gene could be determined by
139 direct sequencing. The sequences have been deposited in the DNA Data Bank of
140 Japan (DDBJ) with accession numbers AB757860 and AB757862 for the 18S rRNA,
141 and AB757865 for 28S rRNA genes, respectively. Two different 18S rRNA gene

142 sequences were obtained from one oocyst suggesting two alternative possibilities.
143 Coccidian oocyst is formed from diploid zygote. During sporogony, eight haploid
144 sporozoites are produced as the result of meiosis and mitosis. The two individual
145 sequences might be derived from each haploid genome of sporozoites within one
146 oocyst. The other possibility is the heterogeneity of the 18S rRNA genes existing in
147 the haploid genome of *Isospora* sp. It has been suggested that the rapid
148 birth-and-death evolution of the apicomplexan genome generated multiple
149 heterogeneous copies of the 18S rRNA gene in the haploid genome [11]. Ikarashi *et*
150 *al.* (2013) discovered that *Cryptosporidium andersoni* carries two different
151 genotypes heterogeneously in its haploid genome [12].

152 There were other eimerian oocysts in the same pigeon-stool specimen. We
153 also isolated more than 15 eimerian oocysts, performed the single-oocyst PCR and
154 sequencing (Matsubara *et al.*, Unpublished). The 18S rRNA gene sequences obtained
155 from *E. labbeana* showed high homology (99%; 1218–1227 nucleotides were
156 identical among 1229 bp) to the available sequence of *E. labbeana*-like oocyst
157 (KT305927) [13]. These sequences of the coexisting pigeon *Eimeria* were not

158 identical to the two sequences from *Isospora* sp. This evidently indicated that the
159 *Isospora* was not insufficiently or abnormally sporulated oocyst of *Eimeria*
160 coexisting in the pigeon-stool specimen.

161 Multiple alignment analyses of the 18S rRNA gene were performed using
162 both Clustal-Omega [14] and MUSCLE [15] in the SeaView 4.6 application [16],
163 and the MUSCLE alignment generated superior results. Gaps and ambiguous
164 positions were edited and eliminated manually. After editing, 1,162 nucleotides of
165 the 18S rRNA gene remained and were subjected to a phylogenetic analysis. A
166 maximum likelihood (ML) tree was constructed using RAxML v8.1.17 [17] with the
167 GTRGAMMAI substitution model (a thorough bootstrap analysis was conducted). In
168 total, 1,000 tree replicates were generated to evaluate bootstrap support. The
169 constructed tree is depicted in Fig. 2A. Pigeon *Isospora* sequences 1 and 3, located
170 beside the *Isospora/Atoxoplasma* clade, formed a monophyletic clade but were not
171 identical to other known species, indicating that the sequences of pigeon *Isospora* are
172 distinct from the others. However, the *Isospora/Atoxoplasma* clade was not strongly
173 supported by bootstrap values. Also, we could not determine the relationships of

174 operational taxonomic units (OTUs) within the *Isospora*/*Atoxoplasma* clade due to
175 the low bootstrap support throughout. Nevertheless, *E. labbeana* and pigeon
176 *Isospora* belonging to the different clades again indicates that pigeon *Isospora* was
177 not the insufficiently or abnormally sporulated oocyst of *E. labbeana*.

178 For the 28S rRNA gene, the sequence from the isolated *Isospora* oocysts
179 (using the same oocyst that provided the 18S rRNA information) did not show
180 heterogeneity. The ML tree based on the 28S rRNA gene sequences was constructed
181 using the same method as the 18S rRNA gene tree, and the MUSCLE alignment
182 provided superior results. The constructed tree is depicted in Fig. 2B. The overall
183 branch lengths are short, suggesting that this locus is not suitable for analyses of the
184 phylogenetic relationships of *Isospora*.

185 Based on our results, we cannot clearly discuss the phylogenetic
186 characteristics of pigeon *Isospora*. Other loci, including mitochondrial COIa and
187 COIb [18, 19], or other protein-coding genes may help increase the phylogenetic
188 resolution. In this study, we attempted to amplify these sequences but the trials were
189 unsuccessful, probably due to limitations of the template genome. Further

improvement of whole-genome amplification techniques may increase the efficacy of PCR amplification and enable us to improve our understanding of *Isospora*.

Mandour *et al.* (1986) described *Atoxoplasma columbae* as a parasite from Columbiformes in Assiut, Egypt [20]. The genus *Atoxoplasma* was previously recognized as a blood parasite and was recently divided into two groups: one transmitted by mites considered to be a synonym of *Lankesterella* and the other with fecal-oral transmission as a synonym of *Isospora* [21, 22]. In the original description of *A. columbae*, only a rough line drawing of intra-erythrocyte merozoites from the thin blood smear of a pigeon was available; none of the morphological features of other stages were recorded, and no molecular information is available. In particular, the lack of oocyst information raises the question of whether *A. columbae* can be transmitted via an insect vector or fecal oocyst; in the latter case, it would be recognized as *Isospora*. In the present study, we did not make blood smears or perform a dissection to clarify the infection site of the pigeon *Isospora* sp. Hence we only mention the possibility that the detected pigeon *Isospora* sp. is *A. columbae*.

205 In conclusion, we detected an isosporoid oocyst from the domestic pigeon *C.*
206 *livia*. It had distinct morphology compared with other species isolated from the same
207 host order. This is the first case in which an *Isospora* sp. has been reported from *C.*
208 *livia* worldwide, and that a coccidium has been reported from Columbiformes in
209 Japan.

210

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215 the Kuribayashi Science Foundation.

216

217 **Ethical standards**

218 All experiments herein comply with the current laws of Japan.

219

220 **Conflict of interest**

221 The authors declare that they have no conflicts of interest.

222

223 **References**

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314

315 **Figure legends**

316 **Fig. 1. Morphology of the pigeon *Isospora* sp. isolated in this study**

317 (A) Differential interference contrast image of a sporulated oocyst of the pigeon

318 *Isospora* sp. (B) Line drawing of sporulated oocysts of the pigeon *Isospora* sp. Note

319 the obvious sub-Stieda bodies. Bar: 10 μ m.

320

321 **Fig. 2. Phylogenetic tree of *Isospora* sp. and related taxa**

322 (A) A maximum likelihood (ML) phylogenetic tree of the pigeon *Isospora* sp. and

323 related taxa was constructed using 1,162 bp of the 18S rRNA gene. The confidence

324 in the tree topology is shown with ML nonparametric bootstrap values (values >50

325 are shown). The pigeon *Isospora* sp. is underlined. (B) ML tree of *Isospora* sp. and

326 related taxa is shown. A total of 598 bp of the 28S rRNA gene were analyzed. ML

327 nonparametric bootstrap values (>50) are shown. The pigeon *Isospora* sp. is

328 underlined.